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Abstract

We apply functional genomics techniques to describe gene expression profiles of dopaminergic neurons in the brain, aiming to identify the molecular basis of the vulnerability of selected classes of dopaminergic cells in Parkinson's Disease (PD).
During the first year of research we have established an *in house* cDNA microarray facility with SISSA-RIKEN 20K slides that contain 2033 different full-length cDNAs encoding for secreted proteins. We have also developed a technique to specifically label subgroups of dopaminergic neurons.
At the end of this project we envision the description of the gene expression profiles of dopaminergic cell subgroups identifying new trophic factors for DA neurons. These molecules will represent new potential therapeutics for PD. Furthermore, genes specifically expressed in vulnerable subgroups will be potentially involved in their selective susceptibility, representing ideal targets for drug discovery.

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Annual Report for Award Number W81XWH-04-1-0599

Introduction

We apply functional genomics techniques to describe gene expression profiles of dopaminergic neurons in the brain, aiming to identify the molecular basis of the vulnerability of selected classes of dopaminergic cells in Parkinson's Disease (PD).
PD is the second most common progressive neurodegenerative disorder, affecting 1-2% of all individuals above the age of 65. The selective degeneration of subsets of midbrain dopaminergic neurons is believed to be the primary cause for disruption of the ability to control movements. Although levodopa remains the most effective drug, none of the current available treatments have been proven to slow the progression of the disease (1,2).
Meaningful hypothesis on the causes of PD and the design of new therapeutics must consider reasons and mechanisms of the selective vulnerability of mesencephalic dopaminergic neurons. Cell loss is maximal in the dopaminergic A9 neurons of the Substania Nigra (SN) while the adjacent dopaminergic A10 cells of the Ventral Tegmental Area (VTA) are largely spared. Most importantly, there are significant differences within A9 and A10 cells: lesions are more conspicuous in the large, calbindin-negative A9 neurons that occupy caudal, lateral and ventral positions (3-5). This pattern of cell loss can be recapitulated in experimental mouse models of the disease such as intraventricular administration of 6-OHDA and MPTP intoxication. The biological basis of these differences within dopaminergic cells is unknown (6,7).
Our hypothesis is that genes specifically expressed in A10 neurons are responsible for cell survival. Special attention is dedicated to the identification of secreted factors that may be potential candidates for restorative therapy.
Furthermore, genes specifically expressed in A9 subgroups are potentially involved in their selective susceptibility, representing ideal targets for drug discovery (8,9).

Although idiopathic PD is usually sporadic, the identification of genes associated with rare forms of familial PD has provided novel insights into the molecular mechanisms of pathogenesis (10). The A53T mutation in α-synuclein has been detected in at least twelve families. This protein is a major component of Lewy bodies, fibrillar intracytoplasmic inclusions that are the histopathological hallmark of PD. The derangement of α-synuclein metabolism is believed to contribute to the pathogenesis of sporadic PD. Interestingly, mutant α-synuclein expression in transgenic mice results in its progressive accumulation in neuronal inclusions, loss of dopaminergic terminals in the basal ganglia and motor impairments (11,12).

By the combination of using a line of transgenic mice where dopaminergic neurons are labelled (13) and region-specific injections of retrograde fluorescent dyes, we can identify homogenous sub-groups of mesencephalic DA neurons. After FACS enrichment, labelled neurons are harvested and their mRNA is amplified by SMART7 amplification technique (14). We are planning to use these probes on a SISSA-RIKEN cDNA microarray that contain the large majority of protein-coding genes, including many newly discovered secreted proteins (15-17). The cell-type specific pattern of expression will be confirmed in normal, *post-mortem* human brains.
We will integrate these data with the analysis of cell-type specific-derangements of gene expression provoked by mutant α-synuclein.

Body

The application consisted in two aims:

Aim 1. Expression profiles of A9 and A10 DA neurons and cell vulnerability in PD.

Aim 2. Analysis of gene expression in a mouse model of PD.

Aim 1. Expression profiles of A9 and A10 DA neurons and cell vulnerability in PD.

Background

The mesencephalic dopaminergic cell system contains at least 13 different nuclei that are classified in three groups of projecting neurons according to well-established cytoarchitectural criteria. The nigral neurons (A9) are present in the pars compacta (SNc) and pars reticulata (SNr) of the substantia nigra. Caudally, these cells share borders with the peri- and retrorubral A8 DA cells. A10 DA neurons are mainly confined to the VTA (4,5).
In PD cell loss is maximal in A9 neurons, intermediate in medioventral A10 and A8 cells and it is negligible in the A10 medial group.
Our working hypothesis is that subpopulations of DA neurons may be differentially vulnerable in PD because they normally express different sets of genes.
In this application we propose to apply functional genomics techniques to describe gene expression profiles of DA neurons in different mesencephalic nuclei.
To describe the gene expression profile of DA cells we must: 1. identify subgroups of DA neurons in the living state; 2. amplify mRNA from a small number of cells in a reproducible and sensitive fashion; 3. hybridize aRNA probes to oligonucleotides and cDNA microarrays.

Testing experimental strategies described in the original application.

To isolate DA neurons for gene expression analysis we have tested the three strategies proposed in the original application.

Strategy #1. Single DA neurons are identified in the living state by immunostaining with E6-Cy3 after dissociation of the mesencephalon from PLAP-expressing neurons. Cells are then harvested with a patch electrode. RNA is amplified from a single cell and probed on a microarray slide (13).

Strategy #2. After dissociation, homogeneous groups of cells are identified, harvested and pooled. Microarray analysis is then performed on samples containing 10-20 cells.

Strategy #3. SN and VTA are independently dissected out. PLAP-expressing cells from these two regions are plated in RNAlater (Ambion) and purified by FACS. A9 and A10 DA neurons are then gene profiled on microarrays (18).

We have first established a short-term culture of mesencephalic neurons where solitary DA neurons were identified in the living state by immunostaining with E6-Cy3.

To do so, we obtained from the laboratory of Elio Raviola (Harvard Medical School) the E6 hybridoma cell line. Anti-PLAP E6 antibodies have been produced, purified and directly coupled to Cy3.

We have then dissected P15 mouse brain from the THpPLAP transgenic mice. 1 mm mesencephalic coronal sections were obtained by slicing with an acrylic brain matrice (Stoelting, IL). To enrich for A9 neurons, the most lateral region of the mesencephalon was dissected out under a microscope. The microdissection of the most medial part of the area enriched for A10 neurons. Tissues were then enzymatically digested and mechanically triturated. After a brief centrifugation, cells were resuspended and incubated with E6-Cy3 antibodies and plated.

We consistently obtained mesencephalic short-term cultures with an average of 2-3% DA neurons labeled in the living state.

In applying the different strategies we encountered the difficulties we have predicted in our application.

Strategy 1. We performed on mesencephalic DA neurons the same experiment as in Gustincich et al.; 2004. Single labeled neurons were patch-clamped and harvested. aRNA were synthesized using the SMART7 technique. 19 cycles of PCRs and two rounds of T7 linear amplification were used. Probes were tested for the presence of TH cDNA and hybridized on SISSA-RIKEN cDNA microarrays. Unfortunately, single to single cell reproducibility was low.

It was therefore immediately clear that this techniques is useful at the single cell level as a gene discovery method while it is of limited applicability for differential analysis of gene expression.

As shown in Gustincich et al; 2004, we confirmed that the SMART7 technique worked well starting from 100 pg of total RNA suggesting that strategy 2 was more convenient.

Strategy 2. These acute, short-term cultures were characterized after chemical fixation with the limited number of known markers for subsets of DA neurons to correlate cell morphology with expression patterns and to assess the effects of the dissociation procedure on cell size and shape. Our goal was to unambiguously identify large TH-positive, calbindin-negative neurons since they represent the most characteristic target of neurodegeneration. We attempted to harvest three types of DA neurons: large A9 cells (perikaryon larger than 20 μm; calbindin-negative), intermediate-sized A9 cells (~15 μm perikaryon; calbindin-positive) and small A10 neurons (ERβ-positive).

Unfortunately, the distribution of cell sizes and shapes was an ambiguous criteria to identify different types of A9 and A10 DA neurons before fixation and immunostaining. Therefore groups of DA cells were not unambiguously homogeneous.

We also tested antibodies against the extracellular part of N-CAM to label DA neurons in the living state. Our procedure was unsuccessful.

Strategy 3. We previously proposed to consider A9 and A10 cell groups as two homogenous populations since the percentage of neurons that degenerate in PD is in average consistently higher for A9 cells than A10. According to this model we proposed to harvest PLAP-expressing cells from these two regions after FACS purification. Unfortunately, although we separated and dissected out the medial from the lateral region of the mesencephalon, we were never sure that we were indeed enriching by FACS a pure A9 or A10 population.

A new experimental flow-chart

We decided to focus our attention on approaches that allow the identification and harvesting of subgroups of A9 and A10 neurons. As described above, the classification in A9 and A10 is indeed superficial lacking the monitoring of at least 13 subgroups with different susceptibility in PD.

This classification is based on anatomical criteria that treasure in most part the location of cell bodies and, most importantly, the organization of their connections.
These neurons belong to different classes because their patterns of connections are different (i.e. sending processes to different areas of the brain).

Once we can label specific cells subgroups, we can then pool 10-20 neurons that represent a homogeneous population of cells. The RNA content of 10-20 neurons is approximately 100 pg in the range of a reproducible and sensitive amplification by SMART7. Labelled neurons are then purified by FACS.

We have focussed our attention on methods to label specific subpopulations of neurons *in vivo*.

We can consistently label an homogeneous population of DA neurons by stereotaxic injection of fluoro-gold, a fluorescent dye taken up by synaptic endings and retrogradely transported to neuronal cell bodies (18).

The mice are placed in a stereotaxic instrument and 1 ml of a 5% fluoro-gold (Fluorochrome, Denver, Co) solution is monolaterally delivered to the striatum using a CMA microinjection pump. Injections of fluorogold into the striatum retrogradely labelled TH-positive neurons of the SN via the nigrostriatal pathways.

We have performed these experiments on the THpPLAP mice to obtain E6-Cy3 positive neurons with fluorogold labeling.

We are currently optimizing the cell purification step using mesencephalon dissociation, plating in RNAlater and FACS separation.

The new experimental flow chart is therefore the following:

1. Retrograde labeling of DA neurons by stereotaxic Fluoro-gold bead injection into different areas of the brain of the THpPLAP transgenic mouse. We are focusing our attention on striatum, pre-frontal cortex, nucleus accumbens and olfactory tubercle.
2. Labeling of living DA PLAP-expressing cells with E6-Cy3 antibody. i.e. when Fluorogold injection has occurred in the striatum, DA neurons that send processes to the stratum will be double-labeled with E6-Cy3 and Fluorogold.
3. Mesencephalic cell suspension will be fixed in RNAlater and Fluorogold and E6-Cy3 positive cells will be purified by FACS.
4. RNA will be amplified by SMART7 and hybridized to SISSA-RIKEN cDNA microarrays.

For these experiments we are using the SISSA-RIKEN 20K microarrays that contain the largest collections of full lenght cDNAs. Among them, we have bio-informatically identified 2033 secreted proteins with at least 90 new potential neuropeptides (15-17).

Aim 2. Analysis of gene expression in a mouse model of PD.

Mutant α-synuclein may induce in A9 neurons changes in the expression of genes that are responsible for the cell-type specificity of PD. We proposed to use cDNA microarray technology to identify clusters of genes that are induced by the presence of mutant α-synuclein only in A9 neurons.

We are currently breeding α-synuclein A53T over-expressing mice with our PLAP transgenic mouse to obtain the double transgenic line needed for gene expression analysis according to Aim 1.

Key Research Accomplishments

- Establishment of an *in house* cDNA microarray facility with SISSA-RIKEN 20K slides that contain 2033 different full length cDNAs encoding for secreted proteins.
- Establishment of a technique to specifically label subgroups of dopaminergic neurons.
- Establishment of a technique to purify subgroups of DA neurons.
- Breeding the TH-pPLAP transgenic mice with α-synuclein A53T over-expressing mouse line.

Reportable outcomes

- Gene expression profiles of dopaminergic cell subgroups.
- Identification of potential trophic factors for DA neurons.
- Cell specific dysregulation of α–synuclein A53T over-expression

Conclusions

During the first year of the award we have solved many technical problems that have impaired this kind of approaches in the past. The combination of labelling neurons with specific projections with FACS purification allows the identification of homogenous cell populations. SMART7 amplification is very efficient starting from 100 pg of total material and probes can be hybridized on SISSA-RIKEN cDNA microarrays.

Papers have recently been published on the expression profiles of dopaminergic neurons in the mesencephalon (19-21). In this proposal we plan to extend these data to homogeneous subgroups of DA cells within the A9 and A10 cell groups.

To do so we will take advantage of cDNA microarrays that contain the large majority of mammalian secreted proteins. It has been reported that gene expression data appear profoundly different when comparing Affymetrix and cDNA hybridizations suggesting the relevance of integrating published Affymetrix gene expression profiles with cDNAs.

This work will enable the identification of new potential trophic factors for DA neurons and, therefore, new leads for therapeutic treatments.

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